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Zinc deficiency during growth: Influence on renal function and morphology

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Abstract

This study was designed to investigate the effects of moderate zinc deficiency during growth on renal morphology and function in adult life. Weaned male Wistar rats were divided into two groups and fed either a moderately zinc-deficient diet (zinc: 8 mg/kg, $n=12$) or a control diet (zinc: 30 mg/kg, $n=12$) for 60 days. We evaluated: renal parameters, NADPH-diaphorase and nitric oxide synthase activity in kidney, renal morphology and apoptotic cells in renal cortex. Zinc-deficient rats showed a decrease in glomerular filtration rate and no changes in sodium and potassium urinary excretion. Zinc deficiency decreased NADPH diaphorase activity in glomeruli and tubular segment of nephrons, and reduced activity of nitric oxide synthase in the renal medulla and cortex, showing that zinc plays an important role in preservation of the renal nitric oxide system. A reduction in nephron number, glomerular capillary area and number of glomerular nuclei in cortical and juxtamedullary areas was observed in zinc deficient kidneys. Sirius red staining and immunostaining for ⟨alpha⟩-smooth muscle-actin and collagen III showed no signs of fibrosis in the renal cortex and medulla. An increase in the number of apoptotic cells in distal tubules and cortical collecting ducts neighboring glomeruli and, to a lesser extent, in the glomeruli was observed in zinc deficient rats. The major finding of our study is the emergence of moderate zinc deficiency during growth as a potential nutritional factor related to abnormalities in renal morphology and function that facilitates the development of cardiovascular and renal diseases in adult life.

Keywords: Moderate zinc deficiency; Kidney; Renal nitric oxide synthase; Arterial blood pressure

Introduction

Zinc is an essential trace element required by all living organisms for many physiological functions, including growth, development and reproduction. Intracellular zinc is associated with proteins, primarily via complex interactions with cysteines, acting as an integral component of numerous metalloenzymes, structural proteins, and transcription factors (Vallee and Falchuk, 1993; Coleman, 1992). Moderate and marginal zinc deficiency, due to inadequate intakes, is by far more common than severe deficiency, specially in those stages of life when requirements are increased, such as in infants, children and pregnant women in both developing and developed countries (Vallee and Falchuk, 1993; Powell, 2000).

In a previous study we demonstrated that moderate zinc deficiency during growth induced an increase in blood pressure associated with an impaired vascular nitric oxide (NO) system and/or higher systemic oxidative stress (Tomat et al., 2005). Thus, it appears that moderate zinc deficiency may be more than a nutritional risk factor and that an imbalance in zinc bioavailability during postnatal life and growth may be associated with cardiovascular alterations in adult life.

Nitric oxide synthase (NOS), one of the zinc requiring enzyme families, catalyzes the synthesis of NO and L-citruline from L-arginine in the presence of NADPH and O_2. The NOS family consists of three isoforms which are expressed in many tissues, including endothelium and vascular smooth muscle of renal vasculature and specific tubular segments of the nephron.

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The endothelial NOS and neuronal NOS isoforms are constitutive forms, activated by Ca^{2+}-dependent calmodulin binding, while inducible NOS is regulated at transcriptional level by activators such as cytokines (Kone, 2004; Kone and Baylis, 1997; Alderton et al., 2001). X-ray crystallography of all three isoforms of NOS shows a zinc thiolate (ZnS4) cluster formed by a zinc ion coordinated in a tetrahedral conformation with pairs of symmetrically oriented and phylogenetically conserved cysteine residues at the N-terminal oxygenase domain interface. This zinc center is considered to play an essential role in the catalytic activity of this enzyme by maintaining stability of the dimer interface and integrity of the tetrahydrobiopterin binding site (Alderton et al., 2001; Zou et al., 2002; Ghosh et al., 1999).

In the kidney, NO plays prominent roles in the homeostatic regulation of glomerular, vascular, and tubular functions, as well as a variety of fundamental cell functions, including cell proliferation, transcription, and energy metabolism. In the renal vasculature, NO acts not only as a vasodilator, but also has antithrombotic, anti-inflammatory, antiproliferative and antioxidant properties. The renal vascular bed produces a large amount of NO that maintains renal blood flow and glomerular filtration rate. Moreover, it has been reported that NO is involved in the control of sodium and water excretion. However, other factors involved in the regulation of blood flow in the renal medulla and tubular transporters, such as the autonomic nervous system, angiotensin II, and arginine vasopressin hormone also participate in sodium regulation under physiological and pathological conditions (Kone, 2004; Ortiz and Garvin, 2002; Majid and Navar, 2001; Mattson, 2003).

Moreover, several lines of evidence show that zinc is involved in maintenance of membrane physiological properties, in reduction of oxidative stress and in inhibition of apoptosis. The anti-apoptotic effects of zinc have been attributed to the inhibition of the calcium and magnesium-dependent endonucleases, which cause apoptotic DNA fragmentation, and the inhibition of other effectors such as caspase 3 (Truong-Tran et al., 2000, 2001). An additional mechanism, which may underlie the anti-apoptotic effect of zinc, is a decrease in cellular oxidative stress. Zinc can act as an antioxidant by binding to membrane sites that might otherwise bind redox-active metals (such as copper and iron) and by being an essential component of the copper–zinc superoxide dismutase and of the metallothioneins (Powell, 2000; Truong-Tran et al., 2001).

Taking into account that there are not enough reports studying the renal effects of moderate zinc deficiency in growing animals, we investigated the effects of moderate zinc deficiency during growth on the renal morphology and function and on the renal nitric oxide system in adult life in the same experimental model (Tomat et al., 2005).

Materials and methods

Animals and diets

Three-week-old weaned male Wistar rats from the breeding laboratories of the Facultad de Farmacia y Bioquímica (Universidad de Buenos Aires, Argentina) were randomly divided into two dietary groups and fed either a moderately zinc-deficient diet (ZD, 8 mg/kg of zinc, $n=12$) or a control diet (C, 30 mg/kg of zinc, $n=12$) for 60 days. The animal model used in this experiment has been described previously (Tomat et al., 2005).

Diet composition is outlined in Table 1. Both diets had the necessary nutrients, except zinc content in the mineral mix of the ZD diet, to meet rat requirements for growth according to AIN-93 recommendations. Rats were born from mothers that were fed a regular commercial pelleted rat chow, containing the zinc concentration recommended for the period of pregnancy and lactation (Reeves et al., 1993). Animals were allowed food and deionized water ad libitum.

Animals were housed separately in plastic cages in humidity and temperature controlled environment, illuminated with a 12 h light–dark cycle. All the laboratory material was previously washed with nitric acid (20%) and deionized water. Animal care was carried out according to the National Institutes of Health's Guide for the Care and Use of Laboratory Animals (Guide for the Human Care and Use of Laboratory Animals, National Research Council, National Institutes of Health, Publication No. 86-23, Washington, DC, 1985).

After 60 days of dietary treatment, rats were sacrificed by cervical decapitation and both kidneys were immediately removed and weighted. NOS activity and zinc concentration were measured in the left kidney, while morphometric studies, TUNEL assay and immunohistochemical analyses were performed in the right kidney.

Sample collection and analysis

At 15, 30, 45 and 60 days after initiation of the dietary treatment, blood samples were collected from the rats' tails and

Table 1
Composition of the experimental diets (g/kg of diet)

Diet	C	ZD
Potassium caseinate [a]	200	200
Soybean oil	70	70
Mineral mix [b]	35	–
Zinc-free salts mixture [c]	–	35
Vitamin Mix [d]	10	10
Cholin	1	1
Dextrin	684	684
Zinc	0.03	0.008

[a] Potassium Caseinate (Nestlé Argentina S.A.): containing 85.1 g of protein/100 g.

[b] Composition (g/kg mix): Calcium carbonate (357); Potassium phosphate monobasic (196); Sodium chloride (74); Magnesium sulfate 7 hydrate (146.9); Ferric citrate ammonium (6.06); Zinc chloride (1.79); Manganous sulfate 1 hydrate (0.92); Cupric sulfate 5 hydrate(0.63); Potassium iodate (0.0078); Sodium selenate anhydrous (0.1025); Ammonium paramolybdate 4 hydrate (0.008); powdered sucrose (162).

[c] Composition: identical to mineral mix but without Zinc chloride.

[d] Composition (g/kg mix): Nicotinic acid (3); Ca Pantothenate (1.6); Pyridoxine HCl (0.7); Thiamin–HCl (0.6); Riboflavin (0.6); Folic acid (0.2); Vit B12, cyanocobalamin (2.5); Vit E, all-rac-alpha-tocopheryl acetate, 500 IU/g (15); Vit A all-*trans*-retinyl palmitate, 500 IU/g, (0.8); Vit D_3 cholecalciferol, 400 IU/g (0.25); Vit K phylloquinone (0.075); Powdered sucrose (974.655).

animals were placed in plastic metabolic cages in order to collect 24-h urine and feces samples. Urine volume was determined gravimetrically. Plasmatic and urinary creatinine levels were measured by a colorimetric method (Wiener Laboratory). Creatinine clearance was calculated to estimate glomerular filtration rate (GFR). At the end of the dietary treatment, urinary sodium and potassium concentrations were evaluated with an ion analyzer (Tecnolab, Mod. T-412).

Kidney zinc concentration was determined using an atomic absorption spectrophotometer at 60 days after initiation of the dietary treatment (Perkin Elmer Corp. Analytical method for atomic absorption spectrophotometry. Perkin Elmer Corp. Norwalk CT, 1971). The left kidney was dried at 100 °C for 48 h and wet-ashed with nitric acid using also Parr bombs (Sapp and Davidson, 1991). NIST reference material RM 8435 (whole milk powder) was also subjected to identical treatment in order to verify accuracy of the analytical procedures and ashed with each bath of samples to ensure accuracy and reproducibility of mineral analysis.

Determination of NADPH diaphorase (NADPH-d) activity

The left kidney from C and ZD rats was processed by the NADPH-d histochemical method according to Rothe et al. (1998). This technique was used as a histochemical marker of isozyme-independent NOS since it has been demonstrated that NADPH-d activity is inhibited by preincubation with diphenyleneidonium, a potent inhibitor of NOS (Stuehr et al., 1991a,b; Vincent and Kimura, 1992). Observation, OD measurement and photography were performed on a Nikon E400 light microscope (Nikon Instrument Group, Melville, NY). In all cases, special care was taken to ensure simultaneous fixing and processing of control and experimental tissues. The time and temperature of incubation with the reaction mixture were carefully controlled and the samples were randomly processed.

Computed image analysis

The NADPH-d-stained cells from the different groups were measured by using a computerized acquisition and analysis Software (Scion Image Beta 4.02, Scion Corporation, Maryland, USA). The mean of each OD value was calculated from measurement of OD in different tissue areas of the same section and of different sections of the same organ. Each set of OD measurements (control and experimental groups) was performed blindly and under similar light, gain, offset and magnification conditions.

Table 2
Natriuresis, kaliuresis and diuresis in the control diet group (C) and the zinc-deficient diet group (ZD) at 60 days of the dietary treatment

	DC	DB
Natriuresis (mEq/24 h 100 g)	0.6±0.1	0.7±0.1
Kaliuresis (mEq/24 h 100 g)	0.7±0.1	0.7±0.1
Diuresis (mL/24 h 100 g body weight)	3.7±0.5	3.2±0.4

Values represent the mean±S.E.M. Data were analyzed using Student's *t*-test. $n=12$ for each group.

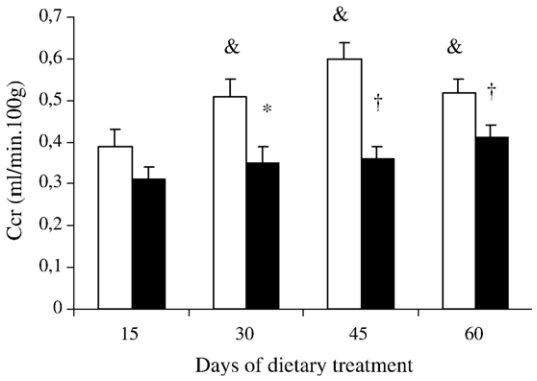

Fig. 1. Creatinine clearance (C_{cr}) measured at 15, 30, 45 and 60 days of the dietary treatment in the control diet group (C, $n=12$) and the zinc-deficient diet group (ZD, $n=12$). Values are means±S.E.M. Data were analyzed by two-way ANOVA, followed by a Bonferroni multiple-comparison post hoc test. Factor treatment (diet): significant effect ($p<0.001$), Factor time: significant effect ($^{\&}p<0.05$ vs. day 15), Interaction treatment x time no significant effect ($p=0.1421$). Significantly different means between C and ZD rats at one time ($^{*}p<0.05$, $^{\dagger}p<0.001$).

Determination of NOS activity

NOS activity was measured in the renal medulla and cortex of the control and the ZD animals using [U-14C] arginine as substrate, as described previously (Costa et al., 2004a,b). Tissue slices (2–3 mm thick) were incubated 30 min at 37 °C in Krebs solution with 0.5 Ci/mL [14C] L-arginine after carbachol (CC)

Table 3
Kidney weight/body weight ratio and morphometric parameters in juxtamedullary and cortical renal tissue of zinc deficient (ZD) and control (C) rats at 60 days of dietary treatment

	C	ZD
Kidney/body weight (%)	0.70±0.05	0.65±0.03
Nephron number (per area)		
Cortical area	11.7±0.4	10.1±0.4$^{\&}$
Juxtamedullary area	6.1±0.4	4.4±0.2*
Total glomerular area (μm²)		
Cortical area	10,103±292	8143±385*
Juxtamedullary area	15,075±736	12,097±911$^{\&}$
Glomerular capillary area (μm²)		
Cortical area	7522±233	5501±298*
Juxtamedullary area	9333±448	6613±471*
Glomerular nucleus number (per area)		
Cortical area	635±29	429±26*
Juxtamedullary area	362±26	158±12*
Glomerular capillary area/total glomerular area (%)		
Cortical area	74.5±1.1	67.3±1.5*
Juxtamedullary area	67.2±1.7	54.9±1.8*
Renal filtration surface area (μm²)		
Cortical area	90,942±3272	79,535±2749*
Juxtamedullary area	60,142±2416	28,925±1280*

Values represent the mean±S.E.M. Data were analyzed using Student's *t*-test. $^{\&}p<0.01$ vs. C; $^{*}p<0.001$ vs. C. $n=12$ for each group.

addition. The amount of [14C] L-citrulline was determined with a liquid scintillation counter (Wallac 1414 WinSpectral, EG&G Company, Turku, Finland). NO production (measured as pmol of [14C] citrulline) in each tube was normalized to the weight of the tissue slices incubated with the substrate during equal (30 min) periods of time and thus expressed in pmol of citrulline/g wet weight of tissue.

Histological evaluation and immunolabeling

Decapsulated right kidneys were cut longitudinally, fixed in phosphate buffered 10% formaldehyde, pH 7.2, embedded in paraffin wax and cut to a thickness of 3 μm. Renal tissue sections were stained with hematoxylin and eosin, periodic acid-Schiff reagent and Masson's trichrome. Ten consecutive cortical and juxtamedullary areas from two renal sections per animal were examined. The number of glomeruli was measured at 1×100 magnification and the area of each field was 1.453 mm^2. The total glomerular area, the glomerular capillary area, the number of nuclei in the glomerular capillary area and the ratio glomerular capillary area/total glomerular area of cortical and juxtamedullary nephrons were determined at 1×400 magnification, each field corresponding to an area of 1.367 mm^2. The filtration surface area, estimated per area of renal juxtamedullary and cortical tissue, was calculated as the product of mean glomerular capillary area by the number of glomeruli per area. The number of nuclei per glomerulus was multiplied by the number of glomeruli and expressed per area of field (1.453 mm^2).

Kidney sections were subjected to ⟨alpha⟩-Smooth muscle actin (α-SMA) and collagen III immunohistochemistry and Sirius red staining (Junqueira et al., 1979) to determine the presence of fibrosis in the renal cortex and medulla. For immunohistochemistry, the following antibodies were used: anti mouse-α-SMA (Sigma Chemical Co., MO USA) and anti-mouse Collagen III monoclonal human antibody, concentrated MU167-UC Clone HWD1.1 (Biogenex, Canyon Road San Ramon, CA 94583, USA). Immunolabeling of specimens was carried out by a modified avidin–biotin–peroxidase complex technique Vectastain ABC kit (Universal Elite, Vector Laboratories) and the specimens were handled as described previously (Hsu et al., 1981). Tissue sections were counterstained with hematoxylin. Collagen accumulation was examined in the renal sections with the collagen-specific stain picrosirius red (Sirius-Red 3 in a saturated aqueous solution of picric acid and fast green as a counterstain).

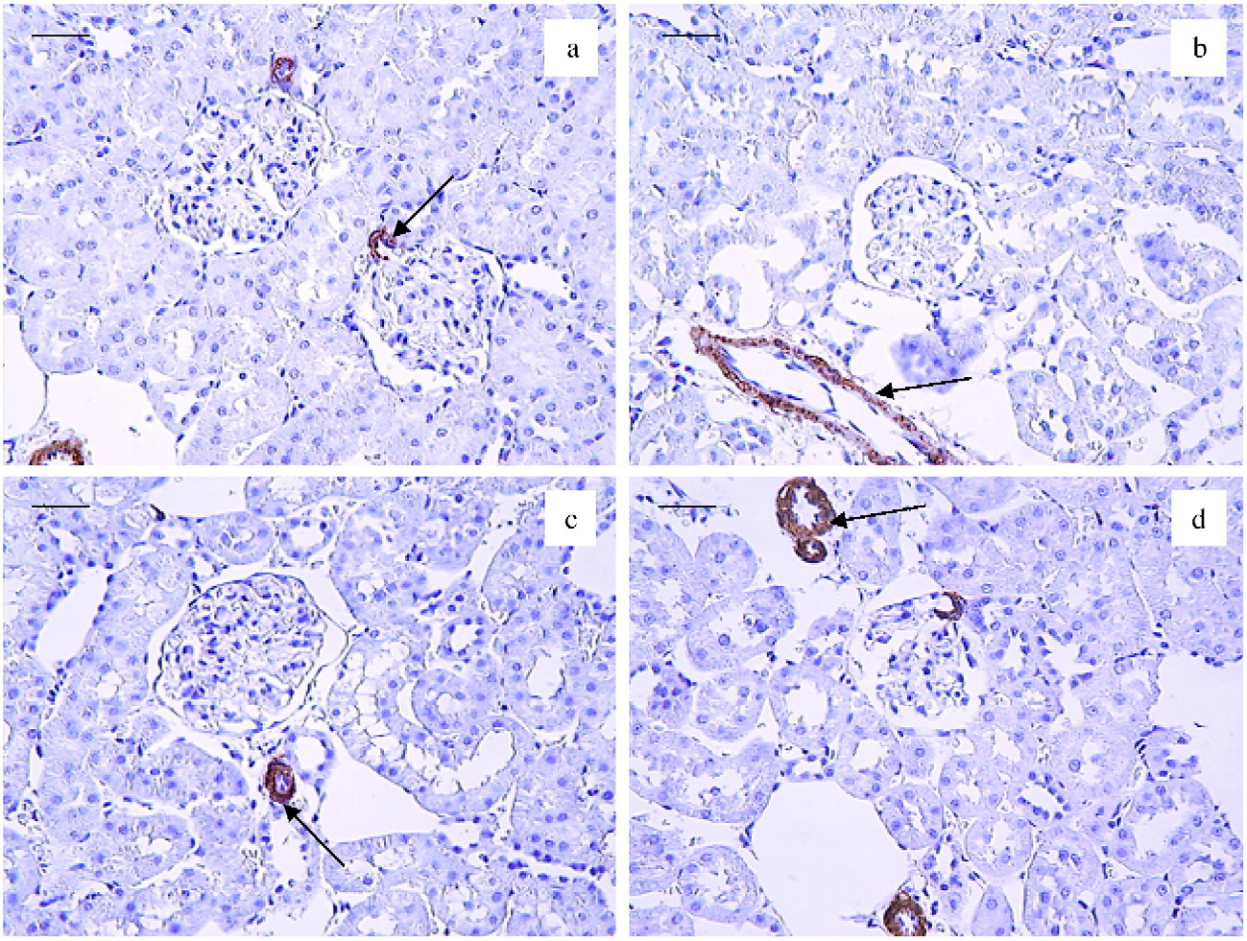

Fig. 2. Immunohistochemical staining for ⟨alpha⟩-Smooth muscle actin in control (C) and zinc deficient (ZD) kidneys. Studies were performed at 60 days of the experimental period in (a) C renal cortical area, (b) ZD renal cortical area, (c) C renal yuxtamedullar area, (d) ZD renal yuxtamedullar area. Arrows indicate positive immunostaining only in vessel wall. All images are at the same magnification of ×400. Scale bar=30 μm.

Histological sections were analyzed using a Nikon E400 light microscope (Nikon Instrument Group, Melville, NY), with the observer blinded to the animal treatment group. Image-Pro Plus 4.5.1.29 software (Media Cybernetics, LP, Silver Spring, MD) was used to evaluate glomerular areas and fibrosis.

In situ detection of DNA fragmentation

The TUNEL assay was performed to detect apoptosis as a marker of cell death. The DeadEnd™ Colorimetric TUNEL System (Promega Corp., USA), a non-radioactive kit designed to end-label the fragmented DNA of apoptotic cells, was used. Paraffin blocks of renal tissue were sectioned at 4-μm thickness and mounted on slides. Renal paraffin sections were deparaffinized in xylene and rehydrated in graded ethanols and PBS. Tissue sections were fixed by immersing the slides in 4% paraformaldehyde solution for 15 min, and incubated in 20 μg/mL proteinase K for 15 min for permeabilization. After refixing tissue sections in 4% paraformaldehyde solution for 5 min, the slides were incubated in the equilibration buffer for 10 min. These procedures were all performed at room temperature. The sections were then treated with the biotinylated nucleotide mix and the terminal deoxynucleotidyltransferase (TdT) enzyme in the reaction buffer for 60 min at 37 °C inside a humidified chamber to allow the end-labeling reaction to occur. Endogenous peroxidase activity in the kidney sections was blocked by incubation for 5 min with 3% H_2O_2 in phosphate buffered saline.

After treatment with streptavidin–horseradish peroxidase solution for 30 min, the sections were stained with diaminobenzidine chromogen and hydrogen peroxide for 10 min. Tissue slides were counterstained with Haematoxiline stain and mounted in an aqueous medium (10% glycerol) for light microscopy. Positive and negative controls for TUNEL stain were performed. A histological section of a control rat kidney was treated with DNA-nuclease before the TUNEL stain was performed, and the vast majority of the cells exhibited nuclear staining. This DNA-nuclease-positive control confirmed that the permeabilization and the labeling reaction had worked correctly. In contrast, a histological section of kidney stained with the TUNEL stain in absence of TdT enzyme (negative control) showed absence of nuclear staining and presence of the counterstain in all of the cells. The number of TUNEL-positive cells per cortical area was counted in 20 visual fields (magnification 1 × 400) for each rat using a Nikon E400 light microscope (Nikon Instrument Group,

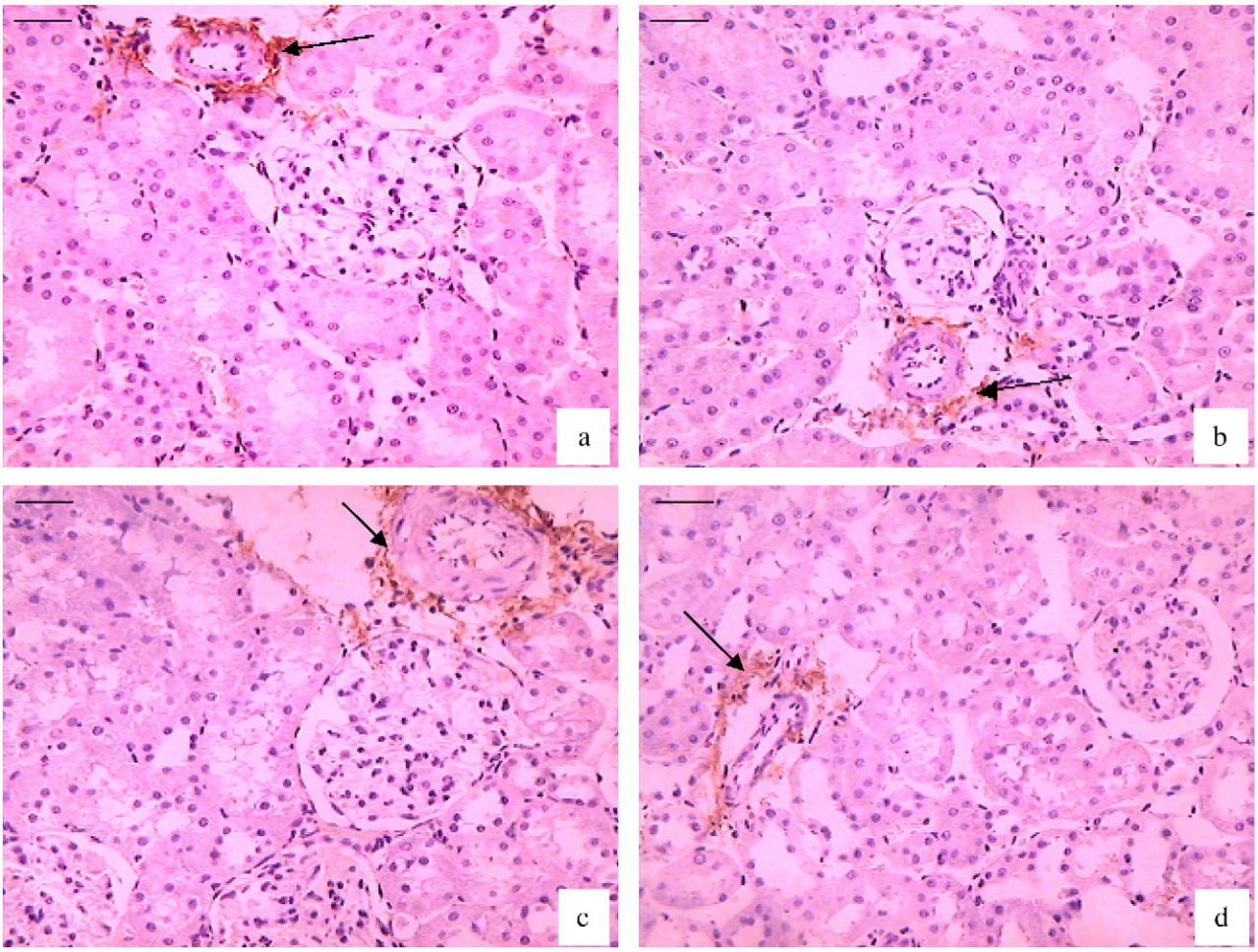

Fig. 3. Collagen III immunohistochemical staining of control (C) and zinc deficient (ZD) kidneys. Studies were performed at 60 days of the experimental period in (a) C renal cortical area, (b) ZD renal cortical area, (c) C renal yuxtamedullar area, (d) ZD renal yuxtamedullar area. Arrows indicate positive immunostaining only in vessel wall. All images are at the same magnification of ×400. Scale bar=30 μm.

Melville, NY) equipped with a digital camera connected to the Image-Pro Plus 4.5.1.29 software (Media Cybernetics, LP, Silver Spring, MD). The measurements were performed blindly and under similar light, gain, offset and magnification conditions.

Statistical analysis

All values are expressed as means ± S.E.M. Prism (Graph Pad Software, Inc., San Diego CA, USA) was used for statistical analysis. Data were analyzed using Student's *t*-test and two-way analysis of variance followed by a Bonferroni multiple-comparison post hoc test. Linear regression analysis was used to determine the relationship between glomerular number and GFR, systolic blood pressure and GFR, systolic blood pressure and number of glomeruli and NOS activity in renal cortex and GFR after 60 days of the beginning of the dietary treatment in all animals. p value of <0.05 was considered a significant difference.

Results

As reported previously, rats fed a moderate zinc-deficient diet showed a significant fall in plasmatic, urinary and fecal levels, indicating that the diet was successful to induce a moderate zinc deficiency (Tomat et al., 2005). Moreover, kidney zinc concentration was lower in ZD rats compared with C rats (C = 27.63 ± 1.99 vs. ZD = 22.40 ± 0.77* μg/g tissue, *$p<0.01$).

We previously showed that ZD diet group exhibited higher levels of systolic blood pressure from day 30 (C: 114 ± 4 vs. ZD: 131 ± 3 mm Hg, $p<0.001$) up to the end of dietary treatment compared with the C group (C: 122 ± 3 vs. ZD: 150 ± 3 mm Hg, $p<0.001$) (Tomat et al., 2005).

There were no significant differences in 24-h urine volume, natriuresis and kaliuresis between the two groups of rats at the end of the dietary treatment (Table 2).

The development of GFR during the dietary treatment is illustrated in Fig. 1. Control animals showed an increase in GFR during the entire age period studied. This is in accordance with previous findings showing that all renal hemodynamic parameters increased in rats from 17 to 60 days old (Aperia and Herin, 1975). However, this raise in GFR was not clearly observed in ZD animals. Moreover, GFR levels were significantly lower in the ZD group than in C rats at 30, 45 and 60 days after initiation of the dietary treatment. In addition, an inversely correlation was observed between systolic blood pressure and GFR ($r=0.7639$,

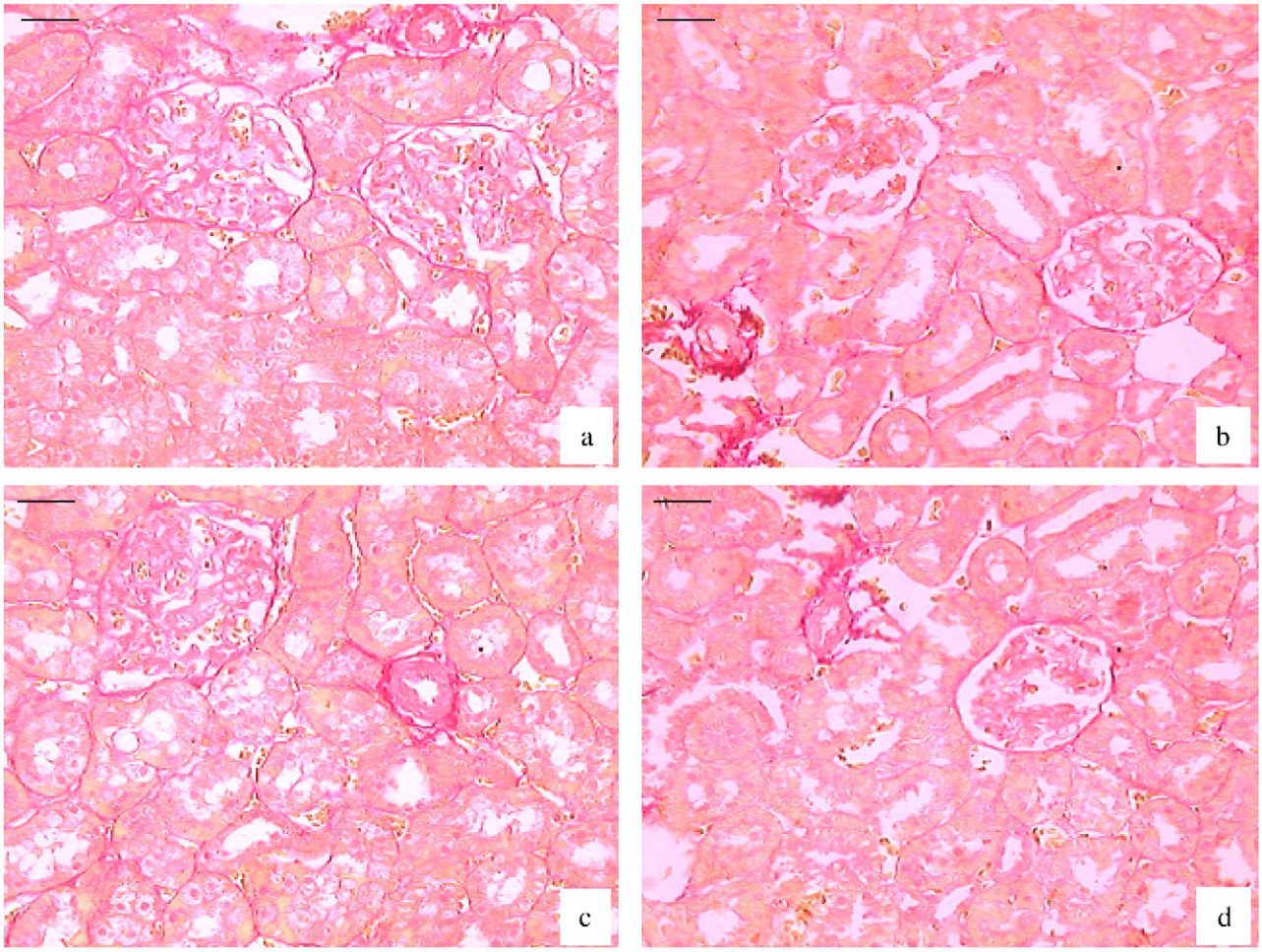

Fig. 4. Sirius red staining of control (C) and zinc deficient (ZD) kidneys. Studies were performed at 60 days of the experimental period in (a) C renal cortical area, (b) ZD renal cortical area, (c) C renal yuxtamedullar area, (d) ZD renal yuxtamedullar area. All images are at the same magnification of ×400. Scale bar = 30 μm.

Table 4
NADPH-d activity measured as optical density (OD) in nephron segments of zinc deficient (ZD) and control (C) rats at 60 days of dietary treatment

Nephron segments	OD	
	ZD	C
Glomeruli	0.151±0.002*	0.172±0.001
Proximal Tubule	0.192±0.002*	0.263±0.006
Henle Limb	0.216±0.005*	0.260±0.004
Distal Tubule	0.193±0.002*	0.265±0.008
Cortical Collecting Tubule	0.200±0.005*	0.259±0.005
Medullar Collecting Tubule	0.199±0.002*	0.252±0.004

Values represent the mean±S.E.M. *$p<0.001$ vs. C. Data were analyzed using Student's t-test. $n=12$ for each group.

$p<0.01$) after 60 days of the beginning of the dietary treatment in all animals.

Kidney weight and morphometric parameters are shown in Table 3. There was no difference in kidney weight, expressed as a percentage of body weight, between the ZD and C groups after 60 days of dietary treatment. However, the cortical and juxtamedullary zone of ZD kidneys showed fewer numbers of glomeruli per area compared with C rats. Moreover, the total glomerular area, the glomerular capillary area, the number of glomerular nuclei and the ratio glomerular capillary area/total glomerular area were reduced in both renal zones studied in the rats exposed to a moderate zinc deficiency during growth. Juxtamedullary and cortical filtration surface areas were smaller in ZD rats than in C ones. A positive relationship was observed between glomerular number and glomerular filtration rate ($r=0.9393$, $p<0.01$) and systolic blood pressure was inversely correlated with number of glomeruli ($r=0.8231$, $p<0.001$) after 60 days of the beginning of the dietary treatment in all animals.

Areas of positive α-SMA and collagen III immunolabeling were confined only to smooth muscle cells of blood vessels of the renal cortex, outer medulla and inner medulla in both groups of rats at day 60 (Figs. 2 and 3). Moreover, light microscopy analysis of Sirius Red stained rat kidney sections confirmed that there were no signs of fibrotic process in glomeruli and peritubular interstitium in ZD rats (Fig. 4).

NOS activity, evaluated by the NADPH-d activity technique, in glomeruli and renal tubules from ZD and C rats at 60 days of the dietary treatment is shown in Table 4 and Fig. 5. NADPH-d

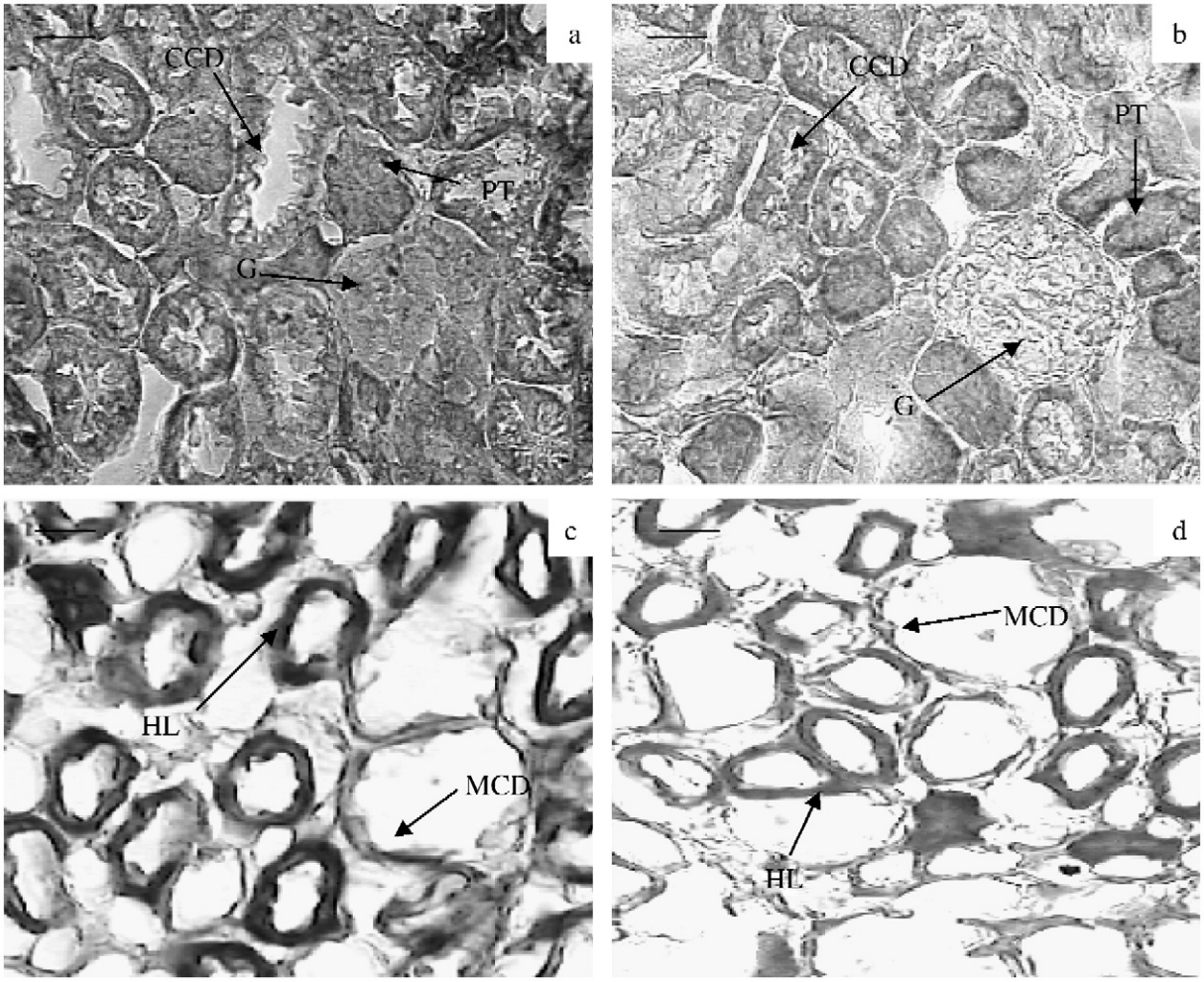

Fig. 5. NADPH-diaphorase staining of control (C) and zinc deficient (ZD) kidney sections. Studies were performed at 60 days of the experimental period in (a) C renal cortex; (b) ZD renal cortex; (c) C renal medulla; (d) ZD renal medulla studied. Arrows indicate staining in glomeruli (G), proximal tubuli (PT), cortical collecting ducts (CCD), henle limb (HL) and medullar collecting ducts (MCD). All images are at the same magnification of ×400. Scale bar=30 μm.

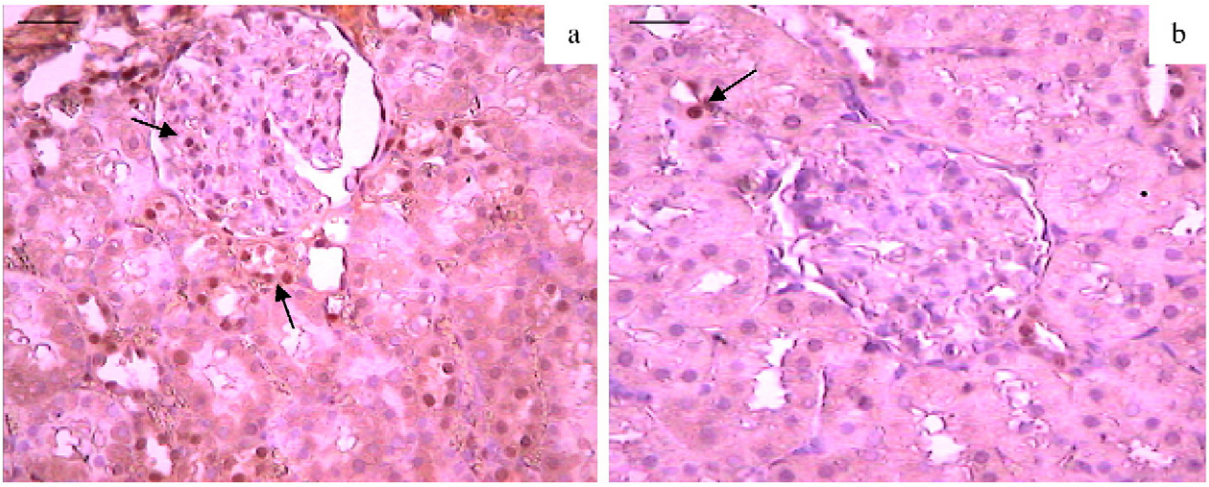

Fig. 6. TUNEL staining of (C) and zinc deficient (ZD) kidney sections. Studies were performed at 60 days of the dietary treatment in (a) ZD renal cortex; (b) C renal cortex. Arrows indicate TUNEL positive cells. All images are at the same magnification of ×400. Scale bar=30 μm.

staining, measured as optical density, was less intense in glomeruli, proximal tubules, henle limb, distal tubules, cortical collecting ducts and medullar collecting ducts of ZD rats compared with the C group.

The data are in accordance with the NOS activity results obtained with the [14C] L-arginine in vitro method in both groups at 60 days of the dietary treatment. The renal medulla and cortex obtained from the animals fed a ZD diet at 60 days of the dietary treatment showed a decreased activity of NOS compared with C (Renal medulla: C=661.07±5.93 vs. ZD=501.75±10.54*; renal cortex: C=510.06±5.29 vs. ZD=403.84±9.08* pmol of citrulline/g wet weight of tissue; *$p<0.001$). Moreover a positive correlation was observed between NOS activity in renal cortex and GFR ($r=0.7149$, $p<0.02$).

Examination of TUNEL-stained renal sections at the end of the experimental period, day 60, revealed an increased number of apoptotic cells in cortical areas of ZD animals compared with controls (C=8.9±1.2 vs. ZD=36.3±2.8*; *$p<0.0001$). The apoptotic cells were localized particularly in distal tubules and cortical collecting ducts neighboring glomeruli and, to a lesser extent, in the glomeruli. Control rats showed little or no TUNEL positive cells in tubules and glomeruli of the cortex (Fig. 6).

Discussion

Multiple factors may induce the development of cardiovascular and renal pathologies. An excess of salt intake, micronutrients deficiency, psychological factors, obesity, endothelial dysfunction and a certain amount of genetic predisposition are generally believed to contribute to the development of cardiovascular and renal diseases. Moreover, there also is evidence showing that environmental factors such as diet during postnatal growth may be of great importance in determining adult blood pressure (Ashton, 2000).

Our results suggest that an imbalance in zinc bioavailability during growth may induce an increase in arterial blood pressure in adult life, probably associated with renal alterations. We reported that the animals, exposed to a moderate zinc deficiency during growth, exhibited higher levels of systolic blood pressure from day 30 after initiation of the dietary treatment compared with the C group, reaching values over 140 mm Hg in the adult stage of life. This increase in arterial blood pressure was associated with a decrease in NOS activity in the endothelium and the smooth muscle of the aorta and arterioles and with a higher systemic oxidative stress (Tomat et al., 2005). The impairment in the vascular NO pathway could alter the normal balance between vasodilator and vasoconstrictor factors leading to an increase in vascular tone and hypertension. In the present study we also showed that the mechanism by which zinc deficiency during growth increased arterial blood pressure may include an altered renal function and an impairment of renal NO system.

Many human studies have reported an association between the changes in zinc metabolism that lead to zinc deficiency and the etiopathogenesis of cardiovascular diseases such as primary arterial hypertension (Bergomi et al., 1997; Tubek, 2005). Moreover, Chiplonkar et al. showed that zinc intake and erythrocyte membrane-zinc were negatively correlated with blood pressure in a traditional Indian lacto-vegetarian population with a great risk of developing mild zinc deficiency (Chiplonkar et al., 2004). However, Sato et al. found that severe zinc deficiency during adult life does not change blood pressure in normotensive rats (Sato et al., 2003), but it can aggravate hypertension in spontaneously hypertensive rats (Sato et al., 2002). On the other hand, Yanagisawa et al. showed that adult rats fed a high zinc diet have higher blood pressure levels than rats fed a standard diet (Yanagisawa et al., 2004). Our results support the fact that zinc deficiency during growth periods would induce evident alterations in arterial blood pressure in the adult life. These controversial results indicate that the effects of zinc deficiency on blood pressure may be related with the duration of the zinc-deficient dietary treatment, the degree of zinc deficiency in the diet, the period of life involved (pregnancy, fetal life, weaning, childhood, adulthood) and the environment.

It is well known that an abnormality in renal function facilitates the development of hypertension (Guyton, 1987;

Ruilope and Rodicio, 2001). Moreover, it has been postulated that a reduction in renal filtration surface area, resulting from a reduction in the number of glomeruli and/or a decrease in filtration surface area per glomerulus, is the fundamental renal abnormality that initiates essential hypertension (Brenner, 2005; Black et al., 2004). In this study, ZD rats did not show the predictable increase in GFR during growth. Moreover, ZD rats presented lower levels of GFR and higher levels of arterial blood pressure than C rats since 30 days of the dietary treatment up to the end of the experimental period. Therefore, we suggest that renal alterations induced by zinc deficiency during growth could explain, at least in part, the mechanisms leading to an increase in arterial blood pressure. Furthermore, this hypothesis is supported by the close inverse correlation observed between GFR and systolic blood pressure.

On the other hand, we showed that the renal dysfunction was associated with renal histological alterations in this model of moderate zinc deficiency. A reduction in number of glomeruli, glomerular capillary area and number of glomerular nuclei in cortical and juxtamedullary zones was observed in ZD kidneys. Therefore, the decrease in filtration surface area in cortical and juxtamedullary nephrons and the highly positive relationship between glomerular number and GFR observed after 60 days of dietary treatment support the fact that lower renal filtration surface area may be responsible for the reduction in GFR. Once more the inverse correlation between number of glomeruli and systolic blood pressure sustain the hypothesis that these renal morphological alterations could be related to the elevated arterial blood pressure levels.

It is known that, in man and rodents, nephrogenesis is completed during fetal and early postnatal life, and that the total number of nephrons established at this time cannot be increased thereafter (Watanabe et al., 1996; Wintour, 1997). Thus, taking into account that nephrogenesis is almost completed at the moment of weaning, we think that the lower number of nephrons observed in the ZD group is not probably due to an alteration in nephrogenesis but to an impairment in normal nephron maturation. It is known that several local factors such as the renin angiotensin system, prostaglandins, growth hormone and insulin-like growth factors play an important role in nephron maturation (Woods and Rasch, 1998; Cingel-Ristic et al., 2004). Therefore, it is probable that some of these factors could be altered in this zinc deficiency experimental model.

Despite these renal histological changes, moderate zinc deficiency during growth generated neither changes in kidney weight, corrected by bodyweight, nor signs of fibrosis in the different structures of the renal cortex and medulla.

Renal alterations were not linked to decreased excretion of water and electrolytes, therefore we suggest that it might be due to adaptive mechanisms induced by different humoral factors involving changes in blood flow in the renal medulla or expression and regulation of tubular transporters (Mattson, 2003).

Therefore, the present data suggest that an adequate content of zinc in the diet may play a significant role in the preservation of renal function. These results support previous reports showing that zinc is important for an adequate renal function and its deficiency could also aggravate renal diseases in adult rats (Yanagisawa et al., 1998).

It is known that NO is an important mediator of renal function since it is responsible for up to one-third of the renal normal blood flow and helps to maintain the low renal vascular resistance under normal conditions. Studies of renal microvasculature responses to NOS inhibition in animal models have shown that the continuous release of NO regulates the resistance of both afferent and efferent arterioles (Kone, 2004; Majid and Navar, 2001). The close correlation observed, in the present study, between GFR and NO production in renal cortex suggests that the decrease in renal NOS activity could be one of the possible mechanisms involved in the decrease of GFR observed.

NO also plays an important role in the control of sodium and water excretion under physiological and pathological conditions since it exerts direct tubular effects on fluid and electrolyte reabsorption (Kone, 2004; Majid and Navar, 2001; Ortiz and Garvin, 2002). The present study showed that zinc deficiency reduced NOS activity in different segments of the nephron tubules at 60 days of the dietary treatment. However, the diminished tubular NOS activity did not provoke perceptible alterations in water and electrolytes excretion. The renal excretory function appears to be the result of the balance between different humoral and hormonal factors that could be altered as a consequence of the zinc deficient diet or secondary to the increase in arterial blood pressure. These speculations could be supported by the fact that zinc is required for structural and functional integrity of multiple transcription factors and enzymes and therefore it is probable that several zinc-requiring proteins may be altered to different degrees in this chronic model of zinc deficiency (Vallee and Falchuk, 1993; Coleman, 1992).

It was reported that zinc is required to promote mitosis and suppress apoptosis; therefore it may serve to coordinate these two opposing growth-regulatory processes (Truong-Tran et al., 2000). The present study found a significant increase in the number of TUNEL-positive cells in cortical tubules and glomeruli of ZD rats exposed to moderate zinc deficiency during growth at 60 days of the dietary treatment. Moreover, the distribution of TUNEL staining demonstrates that most cells of distal tubules and cortical collecting ducts neighboring glomeruli were apoptotic, whereas other tubule sections showed no apoptosis. These findings support previous studies reporting that the distribution and degree of apoptosis susceptibility can vary among cells and tissues (Nodera et al., 2001; Cui and Okada, 2000). The appearance of programmed cell death would depend on the degree of changes in labile cellular zinc pools that are influenced by zinc deprivation (Vallee and Falchuk, 1993; Truong-Tran et al., 2000). According to our results, we hypothesized that a reduction in renal zinc content could induce modifications in intracellular zinc pools of renal cortical cells, increasing the risk of apoptosis and leading to a decrease in the number and size of nephron units, and consequently in glomerular filtration rate. However, more research is necessary to verify the mechanisms of apoptosis induced in this experimental model.

On the other hand, it has been reported that oxidative stress induced by zinc deficiency can reduce NO bioavailability and

trigger renal cell apoptosis (Sato et al., 2002, 2003; Nodera et al., 2001). Sato et al. found that severe zinc deficiency in adult normotensive and spontaneously hypertensive rats induces an increase of superoxide anion production, and consequently a reduction in NO bioavailability (Sato et al., 2002, 2003). These findings support our previous results showing an increase in systemic oxidative stress accompanied by diminished NO system activity in this model of chronic moderate zinc deficiency (Tomat et al., 2005). In addition, NO may be anti-apoptotic in a cell-specific fashion, specially when it is produced in low amounts and at moderate rates in reduced cellular environments (Kim et al., 1999, 2001). Taking in account these previous reports, we hypothesized that an impairment of renal NO system and an increase in oxidative stress could be candidate mechanisms for activation of programmed cell death in kidneys of zinc deficient rats. However, additional studies are required to confirm the induction of oxidative stress in renal tissue in order to corroborate this hypothesis.

In conclusion, the fact that moderate zinc deficiency during growth induces an increase in arterial blood pressure levels associated with alterations in renal function and morphology, as well as in renal NO system activity, supports the hypothesis that zinc deficiency during growth could be a potential nutritional factor in the development of cardiovascular and renal diseases in adult life.

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